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Impact of Bt maize pollen (MON810) on lepidopteran larvae living on accompanying weeds

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Abstract

Environmental risks of Bt maize, particularly pollen drift from Bt maize, were assessed for nontarget lepidopteran larvae in maize field margins. In our experimental approach, we carried out 3-year field trials on 6 ha total. Three treatments were used in a randomized block design with eight replications resulting in 24 plots: (i) near-isogenic control variety without insecticide (control), (ii) near-isogenic control variety with chemical insecticide (Baytroid) and (iii) Bt maize expressing the recombinant toxin. We established a weed strip (20 × 1 m) in every plot consisting of a *Chenopodium album* (goosefoot)/*Sinapis alba* (mustard) mixture. In these strips we measured diversity and abundance of lepidopteran larvae during maize bloom and pollen shed. *C. album* hosted five species but all in very low densities; therefore data were not suitable for statistical analysis. *S. alba* hosted nine species in total. Most abundant were *Plutella xylostella* and *Pieris rapae*. For these species no differences were detected between the Bt treatment and the control, but the chemical insecticide treatment reduced larval abundance significantly. Conclusions regarding experimental methodology and results are discussed in regard to environmental risk assessment and monitoring of genetically modified organisms.

Keywords: Bt maize, insecticide, Lepidoptera, monitoring, nontarget effects, risk assessment

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Introduction

Over the last decade, genes of *Bacillus thuringiensis* (Berliner) ('Bt') that encode lepidopteran-specific protein toxins (Cry1Ab, Cry1Ac and Cry9) were engineered into maize for protection against the European corn borer (*Ostrinia nubilalis* Hbn.) (Fishhoff 1996). However, questions have been raised on the environmental impact of these transgenic plants (Jepson *et al*. 1994; Poppy 2000; Dale *et al*. 2002). In particular lepidopteran nontarget species living on accompanying weeds or weed strips might be affected due to the lepidopteran-specific activity of the toxin (Felke *et al*. 2002). During anthesis pollen covered leaves of host plants are consumed by lepidopteran larvae. This occurs for host plants that grow as weeds within maize fields, and also for host plants that grow in field margins. Field margins are important refugia for some lepidopteran species. As a consequence of the intensification of agricultural practices and the loss of (semi-) natural habitat types, field margins have become increasingly important habitats for conserving biodiversity (e.g. Boatman 1994; Robinson & Sutherland 2002).

Lepidopteran larvae that consume Bt maize pollen from the event Bt 176 or Bt 11, compared to non-Bt control pollen in laboratory studies, had higher mortality, slower development and lower pupae weights (Losey *et al*. 1999; Hellmich *et al*. 2001; Felke *et al*. 2002; Jesse & Obrycki 2002). For MON810 maize pollen, Dively *et al*. (2004) also found an impact on mortality and different fitness parameters for monarch butterfly that consumed pollen throughout larval development in laboratory and semifield trials. Effects of Bt pollen should be dependent on larval species, amount of pollen consumed, type of Bt pollen and growth stage of the

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larvae. However, the extrapolation of laboratory data to the field is somewhat controversial. Laboratory tests provide information on toxicity and fitness parameters, but they often represent 'worst-case scenarios', which do not reflect field conditions or population processes that operate over farming landscapes (Jepson *et al*. 1994; Poppy 2000; Schuler *et al*. 2001). Therefore adverse effects identified in laboratory studies must be verified under field conditions because spatial–temporal and environmental factors can alter possible adverse effects due to, for example, exposure to the toxin or temporal overlap between pollen shed and phenology of lepidopteran larvae (Wolt *et al*. 2003; Poppy & Sutherland 2004).

Risk can be defined as a function of the adverse effect (hazard) and the likelihood of this effect occurring (exposure) (den Nijs & Bartsch 2004). For lepidopteran species the potential hazard is the toxicity of pollen containing Bt toxin and the likelihood of the event is the environmental exposure of lepidopteran larvae to the pollen (Sears *et al*. 2001). Data for the environmental exposure to Bt toxin under field conditions, however, are very rare. In a theoretical approach it was shown that approximately 7% of the German Macrolepidoptera species mainly occur in farmland areas where maize is grown (Schmitz *et al*. 2003). Studies on the monarch butterfly estimated the potential risk under field conditions in the USA. After considering distribution data of the monarch butterfly and their host plants, overlap between anthesis and development of larvae, and exposure of larvae to Bt pollen, risk of Bt pollen to monarch butterflies was determined to be negligible (Sears *et al*. 2001).

The EU directive 2001/18/EC on the deliberate release of genetically modified organisms (GMO) into the environment (EC 2001) outlines environmental risk assessment and monitoring requirements for identifying possible nontarget effects. The main goal of the environmental risk assessment is to identify and evaluate potential effects of growing transgenic plants. The monitoring plan follows two approaches. Case-specific monitoring, the first approach, applies if a risk is identified in the environmental risk assessment. Case-specific monitoring has a more experimental character to evaluate predictable short-term effects. General surveillance, the second approach, is used to monitor for unanticipated adverse effects. General surveillance is not dependent on results from the environmental risk assessment; therefore it is not based on scientific hypotheses. General surveillance is obligatory and is characterized as a general environmental observation (den Nijs & Bartsch 2004; EFSA 2004). However, experimental designs for studying potential effects on nontarget Lepidoptera in the field are still lacking regarding selection of appropriate test species, testing methodologies and statistics (Andow 2003; Dutton *et al*. 2003).

The study aimed to answer the following questions:

1 Does Bt maize (variety MON810) affect nontarget lepidopteran larvae in the field? Similarly, does a conventional insecticide applied to a maize field affect nontarget lepidopteran larvae? How do these treatments compare?
2 Are the methods and experimental design used in this study suitable for environmental risk assessment of potential effects on nontarget lepidopteran larvae under field conditions?

Materials and methods

Experimental design

The presented study was performed from 2001 to 2003. The experimental site consisted of two maize fields (500 m apart) located near Bonn, Germany. One field measured 182 m by 248 m and was divided into 15 plots (Fig. 1); the other measured 178 m by 186 m and was divided into nine additional plots. The size of each plot was about 0.25 ha (56 × 45 m). Both fields were surrounded by conventional maize (variety Romario), minimum 4.5 m strip, for containment purposes. Three treatments were used in a randomized block design with eight replications resulting in 24 plots: (i) near-isogenic variety (variety Nobilis) without insecticide (control, ISO), (ii) near-isogenic variety with chemical

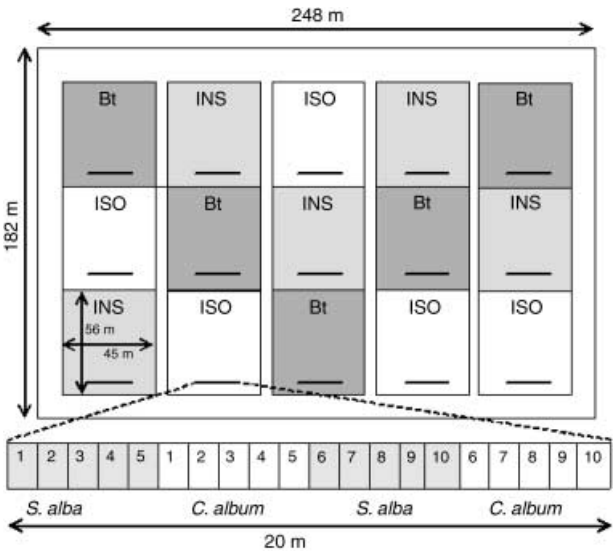

Fig. 1 Experimental design and spatial arrangement of *Sinapis alba* and *Chenopodium album* in weed strips (bold lines). Presented is the randomized block design for one of the two experimental sites. The second field containing nine plots in a similar design was laid out 500 m apart. Fields were surrounded by conventional maize (white area outside the plots). Weed strips are 20 m long. Each square covered an area of 1 m². Abbreviations: ISO: near-isogenic variety (variety Nobilis) without insecticide (control); INS: near-isogenic variety with chemical insecticide application (Baytroid, conventional management); Bt: Bt maize (variety Novelis, event MON810, Bt) expressing the recombinant Cry1Ab toxin.

Table 1 Dates of planting, herbicide application, insecticide application, flowering of maize plants, and samples before and during flowering for the years 2001 to 2003

Year	Planting	Herbicide application	Insecticide application	Flowering period	Sample 1	Sample 2
2001	20.5	29.5	13.7	3–12.8	27–28.7	12–13.8
2002	17.5	10.6	20.7	1–10.8	31.7–1.8	13–14.8
2003	28.4	28.5	11.7	18–30.7	15–16.7	27–28.7

insecticide application (Baytroid, conventional management, INS), and (iii) Bt maize (variety Novelis, event MON810, Bt) expressing the recombinant Cry1Ab toxin. Plots stayed exactly on the same ground in the fields in every year. The fields were conventionally managed according to good agricultural practice. In the three-leaf stage an herbicide treatment (mixture of Callisto 0.9 L/ha and Gardobuc 0.9 L/ha in a water amount of 400 L/ha) was applied to all plots and an insecticide was sprayed in the insecticide plots (Baythroid 50, 750 mL/ha in a water amount of 200 L/ha). Date of insecticide application was dependent on occurrence of the European corn borer (Table 1). A few days after maize sowing, a weed strip of 1×20 m was laid out within each plot. The weed strips were isolated by a minimum of six rows of maize to the next plot to prevent aerially derived input of pollen from neighbouring plots. Minimum distances between two weed strips were about 25 m. During herbicide application the sown weed strips were covered by foil, so that the weeds were able to emerge and survive. Goosefoot (*Chenopodium album* L.) and mustard (*Sinapis alba* L.) were alternately sown every 5 m (Fig. 1). Other upcoming weed species were allowed to grow up to a maximum of 20% coverage, otherwise they were mechanically eradicated. Goosefoot and mustard were chosen because (i) members of these families are typical weeds in maize fields, particularly goosefoot, (ii) these plants host several lepidopteran larvae, and (iii) they grow readily in the field without irrigation.

Measurement of pollen densities

Pollen densities on goosefoot and mustard leaves were estimated at the end of pollen shed (BBCH stage 65, Meier 2001) in 2002. A double-sided adhesive tape was stuck on an microscope slide, then 'leaf prints' were taken from the upper (60–80 cm), middle (40–50 cm), and lower (10–20 cm) third of 20 *C. album* plants, and the upper (40–50 cm) and lower (10–20 cm) part of 20 *S. alba* plants. Leaves of four plants were taken from the different plant heights in five plots each of Bt and near-isogenic maize. Number of pollen grains was counted in 5×0.25 cm^2 squares per leaf under a binocular microscope.

Sampling of lepidopteran larvae

Larvae were sampled by dislodging them into tray (60×45 cm) by either sharply hitting plants with a stick (1 m) or vigorously shaking the plants. Weedy plants were sampled (1 m^2 area, 10 per plot) at the beginning and at the end of pollen shed (Table 1). After identification in the field larvae were carefully put back on the plants. Only a few larvae were not easily identified. They were taken to the laboratory, reared to adult and then identified. Nomenclature was based on Karsholt & Razowski (1996).

Statistical analysis

The primary comparison is between Bt maize (Bt) and the near-isogenic control variety (ISO). According to the risk assessment objective the demonstration of no meaningful change for selected nontarget species in Bt maize relative to the near-isogenic variety should be proven. The use of a nonsignificant P value as a criterion is not appropriate since 'absence of evidence is not evidence of absence' according to Altman & Bland (1995). Therefore the so-called proof of safety (Hothorn & Oberdoerfer 2006), i.e. a proof of equivalence between Bt maize and the near-isogenic variety was performed using two-sided ($1–2\alpha$) confidence intervals according to Chow & Shao (2002). Because many taxa are considered simultaneously, the percentage increase or decrease in abundance of one species is easier for interpretation as the species-specific absolute difference in number of individuals. A ratio > 1 for a taxon is equivalent to an x-fold increase in abundance in the treatment; a ratio < 1 is equivalent to a decrease in abundance in the treatment down to x%. Therefore confidence intervals for ratio's 'Bt/ISO' were estimated. Because the assumption of normal distribution seems to be problematic for these abundance data, nonparametric confidence intervals for the ratios were used. In addition as ties in these count data occur, an exact version was selected. If the confidence interval does contain one, the abundance of a species in the compared treatments can be considered as not significantly changed. Abundance can be different in all three treatments; therefore the ratios INS/ISO and Bt/INS were estimated

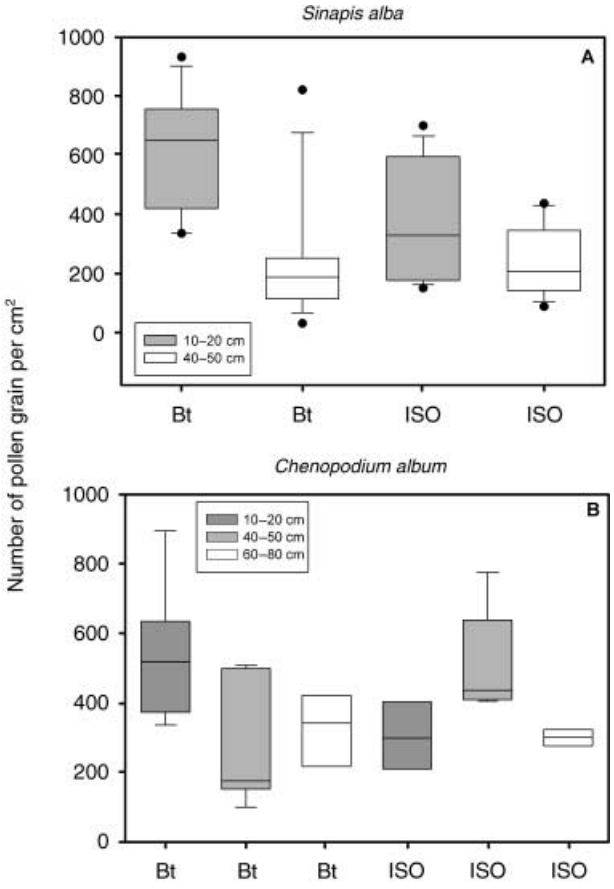

Fig. 2 Merit pollen densities (pollen grains per cm²) on upper, middle and lower leaves of *Sinapis alba* (A) and *Chenopodium album* (B). Presented are median, first and third quartiles (box), 90% range (whiskers) and outliers (points). Results of the statistical analysis are presented in Table 2. Abbreviations: ISO: near-isogenic variety (variety Nobilis) without insecticide (control); Bt: Bt maize (variety Novelis, event MON810, Bt) expressing the recombinant Cry1Ab toxin.

with their common two-sided 90% confidence intervals. The computations were performed by functions written ourselves using the statistical software R (R Development Core Team 2005).

Results

Maize pollen density ranged from 52 to 972 and 100 to 894 pollen grains/cm² for *Chenopodium album* and *Sinapis alba*, respectively (Fig. 2). Pollen deposition from Bt maize was significantly higher on lower leaves for *C. album* (Bt/ISO ratio = 1.77) and *S. alba* (1.87) and on upper leaves for *C. album* (1.13; Table 2). If pollen densities of all heights were pooled for each plant species no significant differences were detected.

Nine lepidopteran species were recovered from mustard plants (Table 4). Only *Plutella xylostella* L. and *Pieris rapae* L. were recurrent in the sown weed strips and abundant enough for statistical analysis. On goosefoot we found the species *Autographa gamma/Macdunnoughia confusa* agg., *Discestra trifolii* Hufn., *Xestia c-nigrum* L., *Lacanobia oleracea* L. and *Phlogophora meticulosa* L. For theses species, numbers of individuals found on all plant species were too low to analyse. In 2002 a preliminary survey showed that the abundance of larvae on goosefoot was also very low. As a consequence goosefoot was not sampled in 2002 and 2003.

Table 2 Results of the 'proof of safety' (confidence interval analysis, Hothorn & Oberdoerfer 2006) comparing pollen densities between Bt maize (Bt) and near-isogenic maize (ISO). Given are the ratio Bt/ISO with the 90% confidence interval. Confidence intervals which do not contain the value one are marked bold

Plant and height	Ratio Bt/ISO	Two-sided 90% confidence interval
C. album (10–20 cm)	**1.77**	**(1.03; 3.46)**
C. album (40–50 cm)	0.47	(0.23; 1.13)
C. album (60–80 cm)	1.13	(0.50; 1.53)
C. album (mean of all heights)	1.03	(0.65; 1.42)
S. alba (10–20 cm)	**1.87**	**(1.11; 2.70)**
S. alba (40–50 cm)	0.96	(0.51; 1.54)
S. alba (mean of all heights)	1.39	(0.95; 2.03)

In all three years the number of lepidopteran larvae of *P. xylostella* and *P. rapae* was lower in the insecticide treatment (Fig. 3). On four sample dates we detected a significant decrease in the number of larvae in the insecticide treatment for *P. xylostella* and on one sample date for *P. rapae* compared to the Bt and control treatments (Table 3). On one sample date a negative impact of Bt maize on lepidopteran larvae was not detected, but at the end of pollen shed 2.72 more *P. xylostella* larvae were found in Bt plots in 2002 (Table 3). In 2001 *P. rapae* larvae tended to be more abundant in Bt plots compared to control plots.

The number of individuals of both most frequent species *P. xylostella* and *P. rapae* on *S. alba* varied between years and sample date within each year. Variation was observed for *P. rapae* between years. In 2001 and 2003 only a few individuals were found on their host plants. In contrast two- to threefold more individuals were detected in 2003 (Table 3). Similar patterns were observed for *P. xylostella*. This species was more abundant in 2001 compared to 2002 and 2003. Variation between sampling dates was clearly demonstrated (e.g. *P. xylostella* in 2001). Larvae were more abundant (661 larvae) during the first sampling before flowering in 2001; 65% of larvae were in the last developmental stage. At the second sampling date most larvae had finished their development and the number of larvae was very low (55). *P. rapae* showed a similar pattern, but had its highest number of larvae in 2002. Results showed that variation of abundance and development of larvae were dependent on species

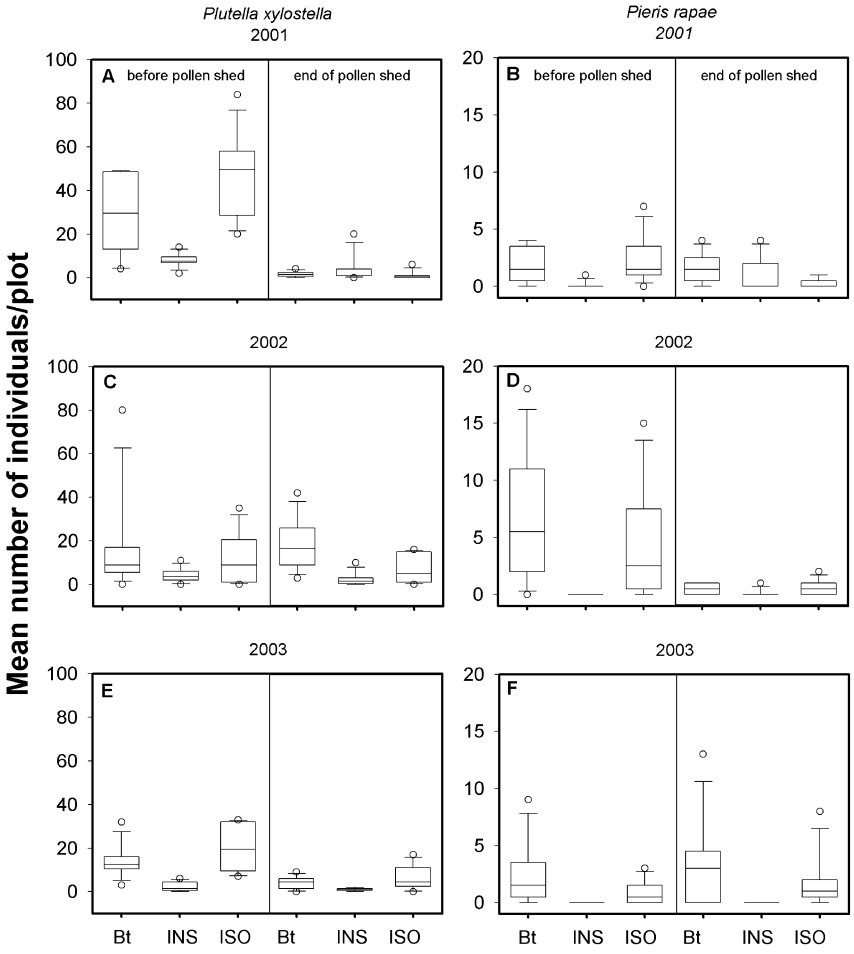

Fig. 3 Number of lepidopteran larvae *Plutella xylostella* (A, C, E) and *Pieris rapac* (B, D, F) for 2001 to 2003. Assessments were made before (left) and at the end of pollen shed (right). Presented is the median of eight plots, first and third quartiles (box), 90% range (whiskers) and outliers (points). Results of the statistical analysis are presented in Table 3. Abbreviations: ISO: near-isogenic variety (variety Nobilis) without insecticide (control); INS: near-isogenic variety with chemical insecticide application (Baytroid, conventional management); Bt: Bt maize (variety Novelis, event MON810, Bt) expressing the recombinant Cry1Ab toxin.

and year. Given these observations most lepidopteran larvae in this study rarely overlapped with maize pollen shed, except for *P. xylostella* in 2001 and 2003, and for *P. rapae* in 2002 (Table 4).

Discussion

Mean pollen densities ranged between 250 and 500 pollen grains per cm^2. However, the single sampling date gives only a rough estimate of level of exposure of larvae to pollen in the chosen experimental design. In several studies mean pollen densities range between 2 and 309 pollen grains per cm^2 on milkweed leaves depending on distance to the field edge (Wraight *et al*. 2000; Pleasants *et al*. 2001; Stanley-Horn *et al*. 2001; Dively *et al*. 2004). Lang *et al*. (2004) published a mean number of pollen grains per cm^2 of about 33 on *Daucus carota* leaves — a lower pollen density compared to the other studies. In this 6-year study relative humidity, growth stage, and distance to maize fields were the most important factors determining pollen density on leaves. Differences between our results and data reported in the literature may have resulted from our experimental design with an extremely high pollen input, because pollen dusted from two sides into the weed strips. Additionally the weed strips were well sheltered from wind and heavy rain by surrounding maize plants. Another reason for the observed differences could be the specific weed plant species. Leave shape and structure (e.g. waxy surfaces or hairs) could affect pollen density on leaves (Lang *et al*. 2004). Differences in the amount of pollen shed by transgenic varieties have not been reported previously. In all cases significantly more pollen per cm^2 was observed in Bt plots. The 2003 summer was extremely hot and dry. Field observations gave the impression that Bt plants grew higher, produced higher biomass and looked healthier than near-isogenic plants under these conditions. As a consequence Bt plants produced more pollen; but these results should be interpreted carefully because of the high data variability. Higher pollen densities in Bt plots, though, would intensify adverse effects on lepidopteran larvae in weeds.

Statistically significant adverse effects of Bt maize pollen on larvae of the most frequent species *Plutella xylostella* and *Pieris rapae* were not found. In contrast significantly higher abundances of *P. xylostella* larvae after pollen shed in 2002

Table 3 Results of the 'proof of safety' (confidence interval analysis) comparing mean number of lepidopteran larvae per plot among the different treatments Bt maize (Bt), near-isogenic maize without (ISO) and with insecticide application (INS) in weed strips. Presented are compared treatments, sampling data, ratio with lower and upper confidence intervals for the most abundant species *Plutella xylostella* and *Pieris rapae*. Confidence intervals which do not contain the value one are marked bold

Compared treatments	Year	Flowering	Ratio	Two-sided 90% confidence interval
P. xylostella				
Bt/ISO	2001	before	0.80	(0.35; 1.43)
INS/ISO	2001	before	**0.27**	**(0.14; 0.72)**
Bt/INS	2001	before	3.00	(0.63; 5.55)
Bt/ISO	2001	during	1.22	(0.71; 2.50)
INS/ISO	2001	during	1.07	(0.50; 4.00)
Bt/INS	2001	during	1.22	(0.38; 2.50)
Bt/ISO	2002	before	1.10	(0.42; 4.50)
INS/ISO	2002	before	0.43	(0.14; 1.71)
Bt/INS	2002	before	2.81	(0.92; 7.00)
Bt/ISO	2002	during	**2.72**	**(1.24; 9.00)**
INS/ISO	2002	during	0.50	(0.13; 2.00)
Bt/INS	2002	during	**5.75**	**(2.73; 14.3)**
Bt/ISO	2003	before	0.71	(0.42; 1.33)
INS/ISO	2003	before	**0.15**	**(0.06; 0.33)**
Bt/INS	2003	before	**5.42**	**(2.20; 12.00)**
Bt/ISO	2003	during	0.76	(0.36; 1.60)
INS/ISO	2003	during	0.32	(0.11; 1.00)
Bt/INS	2003	during	2.58	(1.00; 5.00)
P. rapae				
Bt/ISO	2001	before	1.12	(0.60; 2.50)
INS/ISO	2001	before	0.58	(0.25; 1.60)
Bt/INS	2001	before	2.24	(0.63; 4.00)
Bt/ISO	2001	during	2.24	(1.00; 4.00)
INS/ISO	2001	during	1.41	(0.50; 2.00)
Bt/INS	2001	during	1.73	(0.75; 4.00)
Bt/ISO	2002	before	1.74	(0.64; 4.33)
INS/ISO	2002	before	0.20	(0.06; 1.00)
Bt/INS	2002	before	**6.48**	**(2.00; 19.00)**
Bt/ISO	2002	during	0.82	(0.50; 2.00)
INS/ISO	2002	during	0.82	(0.50; 1.00)
Bt/INS	2002	during	1.41	(0.50; 2.00)
Bt/ISO	2003	before	1.73	(0.75; 3.00)
INS/ISO	2003	before	0.71	(0.33; 1.00)
Bt/INS	2003	before	2.45	(1.00; 6.00)
Bt/ISO	2003	during	1.50	(0.50; 3.00)
INS/ISO	2003	during	0.35	(0.11; 1.00)
Bt/INS	2003	during	3.87	(1.00; 6.00)

and *P. rapae* larvae at the first sample date in 2001 were observed in the Bt plots compared to the near-isogenic plots without the insecticide treatment. Reasons for these results are unclear. One reason could be the observation that near-isogenic maize grew faster compared to the Bt maize in 2002. As a consequence weed strips in Bt plots were more attractive for egg-laying females. In 2001 the near-isogenic maize was highly infested with European corn borer, up to 90% of all plants in a plot; and most plants had broken stalks. Weed strips were less hidden in the maize field and could be easily found by egg-laying females. This was supported by the fact, contrary to expectations, that *P. rapae* larvae were more abundant in the insecticide-treated plots compared to the control plots. Similar results also were observed for *P. xylostella* during maize-pollen shed in 2001.

Other extensive field studies on black swallowtail and monarch butterflies (Wraight *et al*. 2000; Sears *et al*. 2001) that followed laboratory studies where potential effect was identified (Losey *et al*. 1999) reported similar results, where overall risk was low. Possible reasons for these differences between outcomes of field and laboratory studies may be (i) less toxicity of the MON810 pollen used in this study compared to the Bt 176 pollen used in the laboratory studies (Hellmich *et al*. 2001), (ii) field conditions such as rain and wind-reduced pollen deposition (Zangerl *et al*. 2001), and (iii) a lacking temporal overlap between larval development and pollen shed (Oberhauser *et al*. 2001). In particular the last point seems to be most important in the presented study. For *P. xylostella* in 2001 and 2003 and *P. rapae* in 2002 most of the larvae had completed their development (most were last instars) at the beginning of pollen shed. Susceptibility to Bt toxin declines with older instars (Hellmich *et al*. 2001; Felke *et al*. 2002), so the potential for negative impact of Bt pollen is reduced as the larvae grow.

Natural mortality of first instars in the field (e.g. by predators, pathogens or unfavourable weather conditions) was estimated to 41% of the egg stage and 54% of the first instar on average in a review regarding 105 lepidopteran species by Zalucki *et al*. (2002). Therefore it is difficult to deduce harm for lepidoptera species only from laboratory studies. Dively *et al*. (2004) detected a higher mortality and a decreased fitness to monarch larvae consuming MON810 pollen in laboratory and semifield tests. They estimated, however, that these effects on the monarch population were small, ~0.6% to 2.5%; that is lower than natural variability.

Risk estimation of transgenic crops on nontarget lepidopteran species comprises on one hand the toxicity of Bt pollen to each species (hazard) and on the other hand on exposure of larvae to the pollen. Data on toxicity of Bt pollen (Felke *et al*. 2002; Lang & Vojtech 2005) and the possible exposure of larvae on host plant leaves are available (e.g. Wraight *et al*. 2000; Pleasants *et al*. 2001; Stanley-Horn *et al*. 2001; Dively *et al*. 2004; Lang *et al*. 2005; data of this study), but sufficient exposure data on population and landscape level comparable to the studies of Sears *et al*. (2001) or Dively *et al*. (2004) are still missing for most species in Europe. As a consequence the final determinants of risk for lepidopteran species at the population level are incomplete at the moment and further research is needed. Based on the findings from this study and the studies listed above, it seems that European butterfly species are not at high risk.

Table 4 Abundance of lepidopteran larvae on *Sinapis alba*. Presented are experimental year, sample date (year, before flowering = 1, during flowering = 2), number of different instars for each species and the percentage of field strips where the species were observed on both sampling dates (occupancy). Abbreviations: l = last instar, l – 1 last instar minus one, etc

Species	Year	Sample date	l	l – 1	l – 2	l – 3	Σ	Occupancy (%)
Autographa gamma/	2001	1	0	6	11	7	24	62.5
Macdunnoughia confusa agg.	2001	2	2	5	1	0	8	20.8
	2002	1	0	0	2	0	2	8.3
	2002	2	2	2	1	0	5	12.5
	2003	1	0	2	0	0	2	8.3
	2003	2	0	0	1	0	1	4.2
Discestra trifolii Hufn.	2002	1	0	0	1	0	1	4.2
	2003	1	0	0l	0	3	3	4.2
	2003	2	0	0	1	0	1	4.2
Phlogophora meticulosa L.	2001	1	0	1	4	0	5	20.8
	2001	2	0	1	0	0	1	4.2
Pieris brassicae L.	2001	2	5	4	0	0	9	4.2
Pieris napi L.	2002	2	1	0	0	0	1	4.2
Pieris rapae L.	2001	1	6	17	10	2	35	58.3
	2001	2	16	8	0	0	24	45.8
	2002	1	51	34	6	0	91	54.2
	2002	2	1	5	3	1	10	37.5
	2003	1	7	14	6	0	27	45.8
	2003	2	34	8	1	0	43	41.7
Plutella xylostella L.	2001	1	434	182	59	0	675	100
	2001	2	40	12	2	0	54	67.7
	2002	1	203	68	2	0	273	92
	2002	2	207	19	0	0	226	87.5
	2003	1	219	73	2	0	294	96.8
	2003	2	93	1	0	0	94	83.3
Xanthorhoe fluctuata L.	2002	1	0	0	1	0	1	4.2
	2002	2	0	2	1	0	3	12.5
	2003	1	0	1	1	1	3	8.3
	2003	2	0	1	0	0	1	4.2
Xestia c-nigrum L.	2001	1	4	0	1	0	5	12.5

In contrast insecticide effects on lepidopteran larvae in noncrop areas such as adjacent field margin strips or hedges are well described. In particular lepidopteran larvae in field margin strips are endangered to insecticide drift (Davis *et al*. 1991a; Cilgi & Jepson 1995; Snoo & Leeuw 1996; Longley *et al*. 1997; Stanley-Horn *et al*. 2001). Similar to the high pollen exposure, lepidopteran larvae were exposed to the insecticide with the full dose used in field application. Therefore results could not be transferred just to conditions in field margin strips, but are true for weeds within maize fields. However, insecticide drift has the potential to significantly affect insects in noncrop areas (e.g. Davis *et al*. 1991b; Langhof *et al*. 2005).

The presented experimental design can be recommended as a method for testing effects on nontarget lepidopteran species under field conditions despite some general methodological problems. The two most abundant species, *P. xylostella* and *P. rapae*, are potentially good indicator species; however, they are pests on other crops. In the laboratory studies of Felke *et al*. (2002) *P. xylostella* was the most susceptible species to Bt toxin with a calculated LC_{50} of 19.2 consumed Bt176 maize pollen grains. *Pieris brassicae* had a higher LC_{50}, 139.2 Bt pollen grains per cm^2, but was shown to be a good indicator organism in other risk assessment studies, e.g. insecticide drift effects in noncrop areas (Davis *et al*. 1991a, b; Cilgi & Jepson 1995; Longley *et al*. 1997). Not all species are abundant enough for a statistical analysis in the field. In general endangered species will not be included in such an experimental approach because they are too rare under natural conditions (Snoo & Leeuw 1996). This will only be possible if individuals from laboratory rearing were released on the field, but this is very labour intensive and expensive.

High variability of larvae between plots makes statistical analysis difficult. In general variability is a serious problem in field experiments. In this 3-year study, eight replications

for each treatment and 10 subsamples per plot ranks as one of the most intensive field studies of this type. Most studies examining nontarget effects of transgenic crops have used no more than four replications (Marvier 2002). Even with this effort standard statistical tests led to low values in power analyses, which only could be improved with increased replication numbers — a very expensive solution. In contrast, the confidence interval analysis used in this study was able to handle variable data. Limitations of the statistical method such as missing integration of covariables and comparisons between years will be developed in an ongoing research project. Further advantages of the presented experimental design is that high pollen exposure of larvae can be guaranteed, the researcher has only to deal with a manageable number of species through choice of host plants. In contrast other studies evaluated the effects of Bt maize due to diversity and abundance of only adult butterflies which are not directly exposed to the Bt protein (e.g. Lang 2004). The design also offers potential for experimental manipulations as the effects of Bt toxins on larvae could be assessed at the most susceptible developmental stage.

Conclusions

Our experimental design can be a useful element for a tiered environmental risk assessment, which is suggested by different authors (Dutton *et al*. 2003; EFSA 2004; Poppy & Sutherland 2004). In particular the example of nontarget lepidopteran species showed the need for field experiments in a tiered test system. Laboratory and semifield studies often overestimate any adverse effect of Bt maize on lepidopteran larvae. As a consequence field experiments are needed for a comprehensive evaluation. If needed, the experimental design could be expanded for a postmarket environmental monitoring.

Every plant protection measure has an impact on agroecosystems. The overall risk–benefit evaluation needs to compare the impact of both chemical and GM pesticide treatment on nontarget organisms. Our experimental approach demonstrates on one hand the sensitivity of the testing system to agricultural management practice, and on the other hand the environmental impact of conventional and biotechnological pest management strategies. However, interpretation of field data with high variability based on exemplary investigated species needs definition of indicator species (Schmitz *et al*. 2003), for quality of statistical data (Andow 2003) and for threshold values for decision making comparable to the insecticide assessment (Dutton *et al*. 2003).

The presented studies did not indicate any adverse effect of Bt maize to nontarget lepidopteran larvae compared to laboratory and semifield studies. Adverse effects of insecticide application to lepidopteran larvae were detected.

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References

Altman DG, Bland JM (1995) Absence of evidence is not evidence of absence. *British Medical Journal*, **311**, 485.

Andow DA (2003) Negative and positive data, statistical power, and confidence intervals. *Environmental Biosafety Research*, **2**, 1–6.

Boatman N (1994) *Field Margins: Integrating Agriculture and Conservation*. BCPC Monograph No. 58. The British Crop Protection Council, Farnham.

Chow SC, Shao J (2002) A note on statistical methods for assessing therapeutic equivalence. *Controlled Clinical Trials*, **23**, 515–520.

Cilgi T, Jepson PC (1995) The use of tracers to estimate the exposure of beneficial insects to direct pesticide spraying in cereals. *Annals of Applied Biology*, **121**, 239–247.

Dale PJ, Clarke B, Fontes EMD (2002) Potential for the environmental impact of transgenic crops. *Nature Biotechnology*, **20**, 567–574.

Davis BNK, Lakhani KH, Yates TJ (1991a) The hazards of insecticides to butterflies of field margins. *Agriculture Ecosystems and Environment*, **36**, 151–161.

Davis BNK, Lakhani KH, Yates TJ, Frost AJ (1991b) Bioassays of insecticide spray drift: the effects of wind speed on the mortality of *Pieris brassicae* larvae (Lepidoptera) caused by diflubenzuron. *Agriculture Ecosystems and Environment*, **36**, 141–149.

den Nijs HCN, Bartsch D (2004) Introgression of GM plants and the EU guidance note for monitoring. In: *Introgression from Genetically Modified Plants into Wild Relatives* (eds Nijs HCN, Bartsch D, Sweet J), pp. 365–389. CABI Publishing, Cambridge, UK.

Dively GP, Rose R, Sears MK *et al*. (2004) Effects on Monarch butterfly larvae (Lepidoptera: Danaidae) after continuous exposure to Cry1Ab-expressing corn during anthesis. *Environmental Entomology*, **33**, 1116–1125.

Dutton A, Romeis J, Bigler F (2003) Assessing the risks of insect resistant transgenic plants on entomophagous arthropods: Bt maize expressing Cry1Ab as a case study. *Biocontrol*, **48**, 611–636.

EC (2001) Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EC. *Official Journal of the European Communities L*, **106**, 1–39.

EFSA (European Food Safety Authority) (2004) Guidance document on the scientific panel on genetically modified organisms for the risk assessment of genetically modified plants and derived food and feed. *The EFSA Journal*, **99**, 1–94.

Felke M, Lorenz N, Langenbruch GA (2002) Laboratory studies on the effects of pollen from Bt-maize on larvae of some butterfly species. *Journal of Applied Entomology — Zeitschrift für Angewandte Entomologie*, **126**, 320–325.

Fishhoff DA (1996) Insect-resistant crop plants. In: *Biotechnology and Integrated Pest Management* (ed. Persley GJ), pp. 214–227. CAB International, Wallingford, UK.

Hellmich RL, Siegfried BD, Sears MK *et al.* (2001) Monarch larvae sensitivity to *Bacillus thurningiensis*-purified proteins and pollen. *Proceedings of the National Academy of Sciences, USA*, **98**, 11925–11930.

Hothorn LA, Oberdoerfer R (2006) Statistical analysis used in the nutritional assessment of novel food using the proof of safety. *Regulatory Toxicology and Pharmacology*, in press.

Jepson PC, Croft BA, Pratt GE (1994) Test systems to determine the ecological risks by toxin release from *Bacillus thuringiensis* genes in crop plants. *Molecular Ecology*, **3**, 81–89.

Jesse LCH, Obrycki JJ (2002) Assessment of the non-target effects of transgenic Bt corn pollen and anthers on the milkweed tiger moth *Euchatias egle* Drury (Lepidoptera: Arctiidae). *Journal of the Kansas Entomological Society*, **75**, 55–58.

Karsholt O, Razowski J (1996) The Lepidoptera of Europe. *A Distributional Checklist*. Apollo Books, Stenstrup.

Lang A (2004) Monitoring the impact of Bt maize on butterflies in the field: estimation of required sample sizes. *Environmental Biosafety Research*, **3**, 55–66.

Lang A, Ludy C, Vojtech E (2004) Dispersion and deposition of Bt maize pollen in field margins. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz — Journal of Plant Diseases and Protection*, **111**, 417–428.

Lang A, Vojtech E (2005) The effects of pollen consumption of transgenic Bt maize on the common swallowtail, *Papilio machaon* L. (Lepidoptera, Papilionidae). *Basic and Applied Ecology*, in press.

Langhof M, Gathmann A, Poehling HM (2005) Insecticide drift deposition on off-crop plant surface and ist impact on two beneficial non-target arthropods, *Aphidius colemani* Viereck (Hymenoptera, Braconidae) and *Coccinella septempunctata* L. (Coleoptera, Coccinellidae). *Environmental Toxicology and Chemistry*, **24**, 2045–2054.

Longley M, Cilgi T, Jepson PC, Sotherton NW (1997) Measurements of pesticide spray drift deposition into field bounderies and hedgegrows: 1. summer applications. *Environmental Toxicology and Chemistry*, **16**, 165–172.

Losey JE, Rayor LS, Carter ME (1999) Transgenic pollen harms monarch larvae. *Nature*, **399**, 214.

Marvier M (2002) Improving risk assessment for nontarget safety of transgenic crops. *Ecological Applications*, **12**, 1119–1124.

Meier U (2001) *Growth Stages of Mono- and Dicotyledonous Plants*. BBCH Monograph, 2nd edn. Federal Biological Research Centre for Agriculture and Forestry, Braunschweig.

Oberhauser KS, Prysby MD, Mattila HR *et al.* (2001) Temporal and spatial overlap between monarch larvae and corn pollen. *Proceedings of the National Academy of Sciences, USA*, **98**, 11913–11918.

Pleasants JM, Hellmich RL, Dively GP *et al.* (2001) Corn pollen deposition on milk- weeds in or near cornfields. *Proceedings of the National Academy of Sciences, USA*, **98**, 11919–11924.

Poppy GM (2000) GM crops: environmental risks and non-target effects. *Trends in Plant Science*, **5**, 4–6.

Poppy GM, Sutherland JP (2004) Can biological control benefit from genetically-modified crops? Tritrophic interactions on insect-resistant plants. *Physiological Entomology*, **29**, 257–268.

R Development Core Team (2005) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria, www.R-project.org/.

Robinson RA, Sutherland WJ (2002) Post-war changes in arable farming and biodiversity in Great Britain. *Journal of Applied Ecology*, **39**, 157–176.

Schmitz G, Bartsch D, Pretscher P (2003) Selection of relevant non-target herbivores for monitoring the environmental effects of Bt maize pollen. *Environmental Biosafety Research*, **2**, 117–132.

Schuler TH, Denholm I, Jouanin L, Clark SJ, Clark AJ, Poppy GM (2001) Population-scale laboratory studies of the effect of transgenic plants on nontarget insects. *Molecular Ecology*, **10**, 1845–1853.

Sears MK, Hellmich RL, Stanley-Horn DE *et al.* (2001) Impact of Bt corn pollen on monarch butterfly populations: a risk assessment. *Proceedings of the National Academy of Sciences, USA*, **98**, 11937–11942.

Snoo de GR, Leeuw de J (1996) Non-target insects in unsprayed cereal edges and aphid dispersal to the adjacent crop. *Journal of Applied Entomology*, **120**, 501–504.

Stanley-Horn DE, Dively GP, Hellmich RL *et al.* (2001) Assessing the impact of Cry1Ab-expressing corn pollen on monarch butterfly larvae in field studies. *Proceedings of the National Academy of Sciences, USA*, **98**, 11931–11936.

Wolt JD, Peterson RKD, Bystrak P, Meade T (2003) A screening level approach for nontarget insect risk assessment: transgenic Bt corn pollen and the monarch butterfly (Lepidoptera: Danaidae). *Environmental Entomology*, **32**, 237–246.

Wraight CL, Zangerl AR, Carroll MJ, Berenbaum MR (2000) Absence of toxicity of *Bacillus thuringiensis* pollen to black swallowtails under field conditions. *Proceedings of the National Academy of Sciences, USA*, **98**, 11908–11912.

Zalucki MP, Clarke AR, Malcolm SB (2002) Ecology and behavior of first instar larval lepidoptera. *Annual Review of Entomology*, **47**, 361–393.

Zangerl AR, McKenna D, Wraight CL *et al.* (2001) Effects of exposure to event 176 *Bacillus thuringiensis* corn pollen on monarch and black swallowtail caterpillars under field conditions. *Proceedings of the National Academy of Sciences, USA*, **98**, 11908–11912.

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